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Cells are grown in 3-dimensional environments under physiological and pathological condition.
While most of the experiments were done using the monolayer cell culture. Accumulating results showed that the expression patterns of genes and proteins are distinctly different in three-dimensional (3D) cultures when compared with the monolayer (2D) cultures commonly employed. Therefore, we created a novel Polyvinyl alcohol (PVA)3D cell culture system to study human breast cancer cell progression and cancer cell-stromal cell interaction. The results showed that both breast cancer cell (MDA-MB-231)and human fibroblast cell (HFL-1)attachment varied with the different extra cellular matrix components. When the MDA-MB-231 cell invaded into the bottom layer, which contained HFL-1 cell in the PVA 3D cell culture, the cells formed breast cancer tissue-like structure. Furthermore, the MDA-MB-231 cell could be easily identified immunohistochemistry using proper epithelial marker. Therefore, This 3D culture system may be a usefull medol to study cancer cell-stromal cell interaction and cancer cells invasion. The role of the novel MMP-26 in breast cancer invasion using this 3D cell culture system is under investigation.

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Introduction

Proteolytic degradation of the basement membrane (BM) and extracellular matrix (ECM) is a critical process for breast carcinoma cell invasion, and matrix metalloproteinases (MMPs) are thought to play an important role during this process. MMP-26/endometase/matrilysin-2, a novel member of this enzyme family, has been recently cloned and characterized [1]. MMP-26 mRNA is primarily expressed in cancers of epithelial origin, such as breast, endometrial, and prostate carcinomas. More recently, we have found that MMP-26 promoted prostate cancer cell invasion by activating another powerful enzyme, pro-gelatinase B (MMP-9) [2]. Our published data show that the MMP-26 protein is highly expressed in human breast ductal carcinoma *in situ* (DCIS), and in the early stages of human breast ductal invasive carcinoma [3], while little to no MMP-26 protein expression is detected in normal and hyperplastic tissues. MMP-26 mRNA has also been detected in the human breast carcinoma cell line MDA-MB-231. Therefore, we propose that MMP-26 may play an important role in the promotion of breast cancer from DCIS to invasive ductal carcinoma, either through the direct degradation of basement membrane (BM) components, or indirectly through the activation MMP-9.

Reports show that the expression patterns of genes [4] and proteins [5] are distinctly different in three-dimensional (3D) cultures when compared with the monolayer (2D) cultures commonly employed. Polyvinyl alcohol (PVA) is widely used for plastic sponge implants during reconstructive surgery, or as implants for angiogenesis and wound healing studies in animals. PVA sponge is water-tolerant, appropriately porous, compatible with cell growth, and exhibits no cell toxicity [6], making it suitable for use as a 3D matrix in the creation of a novel 3D cell invasion system. The proposed system consists of 3 layers of PVA sponge. The top layer, of 10-15 mm thickness, was used to grow human breast cancer cells (MDA-MB-231), representing the epithelial layer; the middle layer was saturated with ECM components, representing the BM layer; the bottom layer, of 10-15 mm thickness, was used to grow human fibroblast cells (HFL-1), representing the stromal layer. This reconstructed "sandwich" model was then used to study breast cancer cell invasion, and breast cancer cell and human fibroblast cell interactions, during carcinoma progression.

Body

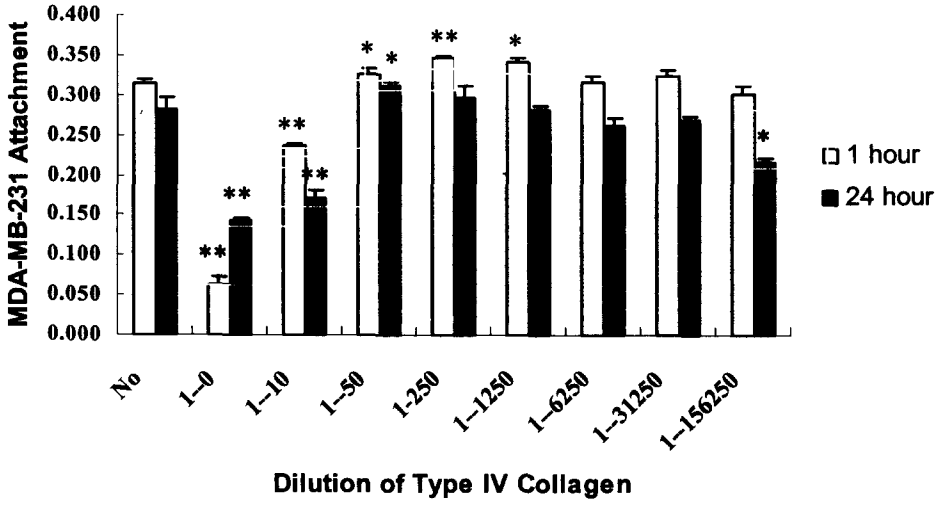
The specific aims of this study are to target MMP-26 in a novel 3D invasion system utilizing functional blocking MMP-26 antibodies and antisense techniques. The following is a summary of the preliminary data generated to date.

1. MDA-MB-231 and HFL-1 cell attachment assays

Growth of the MDA-MB-231 and HFL-1 cell lines in the proposed PVA sponge culture system was attempted, however, the cells did not attached to PVA sponge in an acceptable fashion. Therefore, we attempted to identify which ECM components could enhance cell attachment onto the PVA sponge, and the optimum concentration for these components, as cell attachment to ECM constituents is fundamental to the processes of cancer cell invasion and metastasis. Attachment assays incorporating MDA-MB-231 and HFL-1 cell were performed utilizing 5 different ECM components, including Matrigel (BD Bioscience, Bedford, MA), fibronectin (Gibco Invitrogen Corporation), type IV collagen (Sigma St. Louis, MO), type I collagen (Sigma St. Louis, MO), and laminin (Trevigen). For these assays, 96-well plates were pre-coated with 80 µl of the different ECM components, at varying concentrations, and were then dried overnight in a cell culture with UV decontamination. Cells were than cultured at a concentration of 1.0×10^4 in Dulbecco's Modified Eagle's Medium (DMEM) (LTI) (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin G and 100 µg/ml streptomycin at 37°C in 5% CO_2 for 1 to 24 hours. Cultured cells were then washed three times with PBS prior to staining with

0.1% Crystal Violet for 2 minutes. After the staining solution was removed, absorbance values were read at 492 nm using an Automatic Microplate Reader (Titertek Multiskan MC-340, Flow Laboratories, McLean, Virginia). These experiments were repeated three times, with each iteration yielding similar results, as depicted in **Figure 1.**

A.



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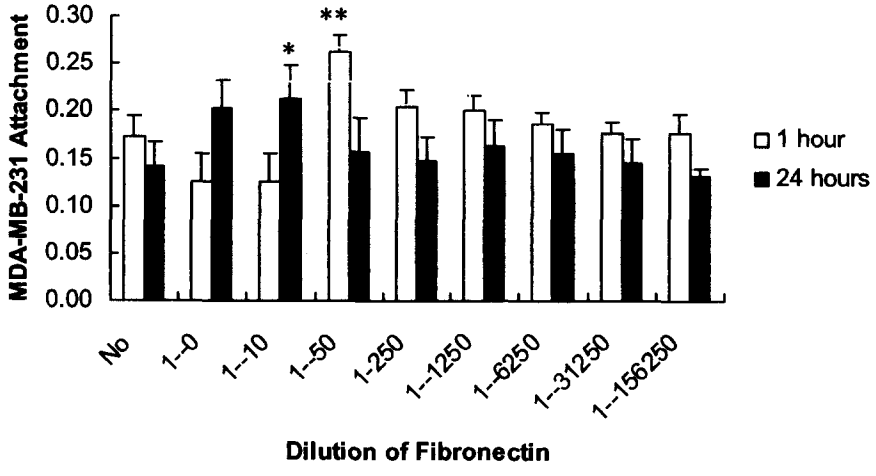

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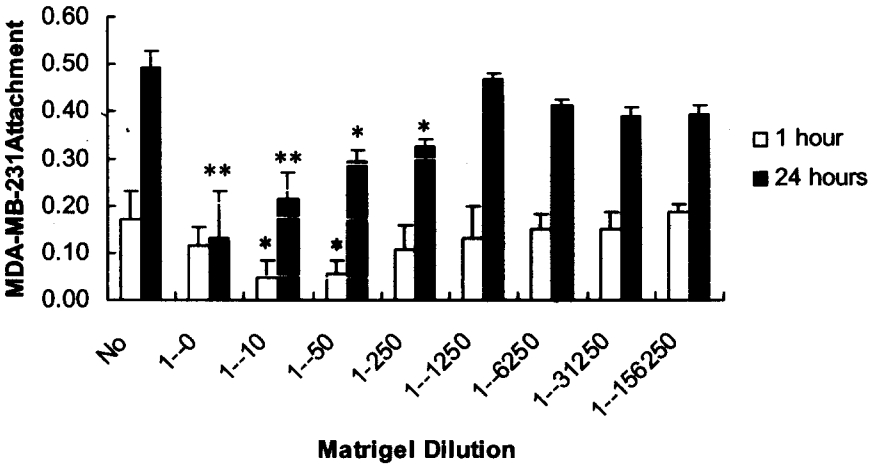
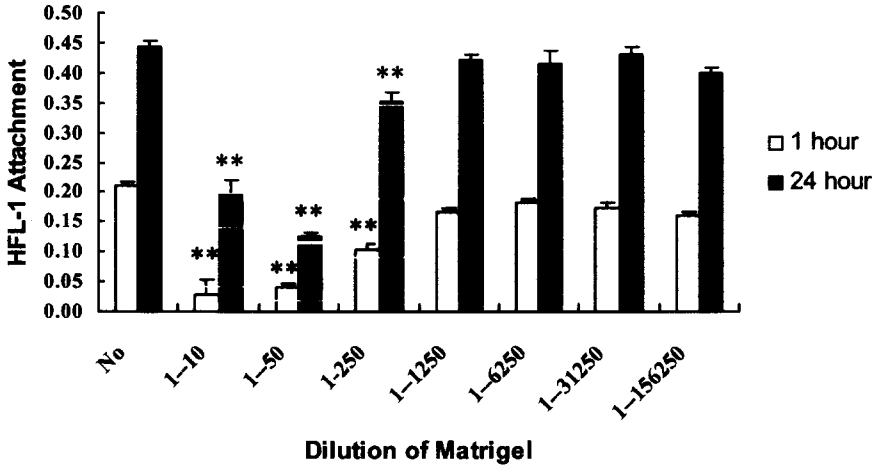
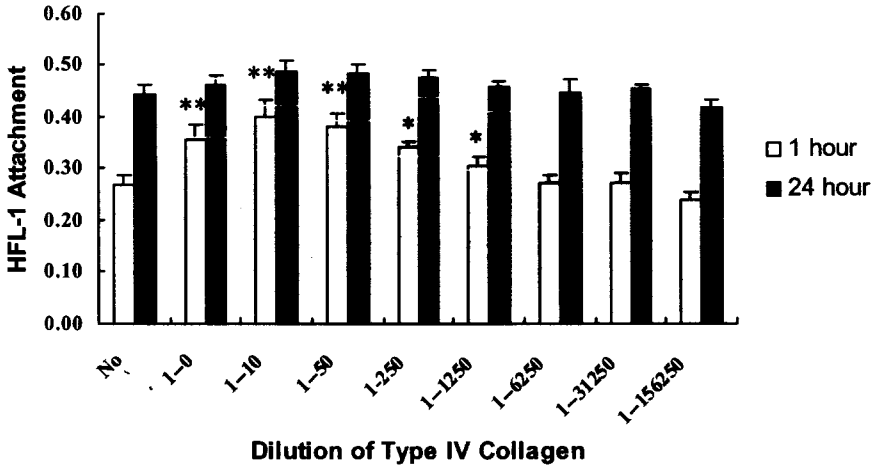

MDA-MB-231Attachment
0.60
0.50
0.40
0.30
0.20
0.10
0.00
1 hour
24 hours
No
1--0
1--10
1--50
1-250
1--1250
1--6250
1--31250
1--156250
Matrigel Dilution

D


HFL-1 Attachment
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1 hour
24 hour
No
1--10
1--50
1-250
1--1250
1--6250
1--31250
1--156250
Dilution of Matrigel

E



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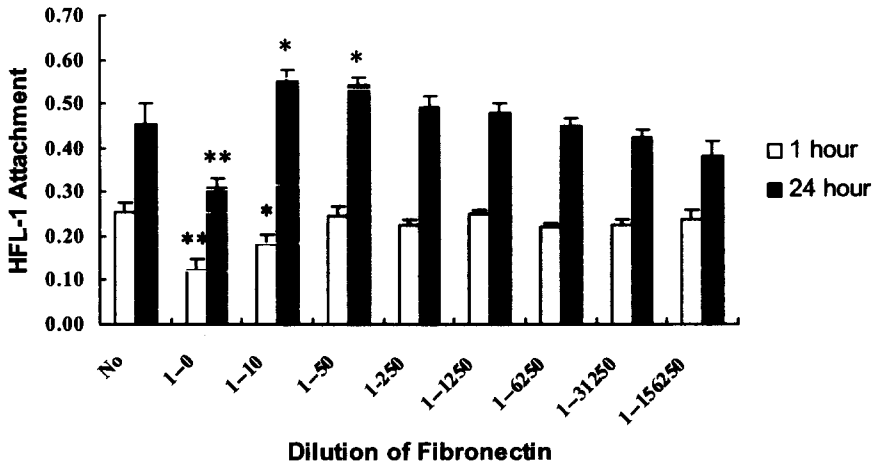

Figure 1. MDA-MB-231 cell and HFL-1 cell attachment on different ECM components. **A-C :** MDA-MB-231 cell attachment on Type IV collagen, fibronectin and Matrigel, respectively. **D-F:** HFL-1 cell attachment on Matrigel, type IV collagen and fibronectin, respectively. The original concentration for type IV collagen and fibronectin is 1mg/ml, and for Matrigel, 13.941mg/ml (laminin is 56%, type IV collagen is 31% and entactin is 8%). Attachment values were derived from OD_{490} values, which were subsequently used for comparative statistical analyses by ANOVA. Statistical analysis of all samples was performed with the least significant difference (LSD) correction of ANOVA for multiple comparisons. Data represent the mean ± standard deviation (SD) from three experiments, where differences with $P<0.05$ were considered to be significant. **: $P<0.01$; * $P<0.05$.

The results of the attachment assays revealed that type IV collagen at 1:250 dilution enhanced the attachment of both MDA-MB-231 and HFL-1 cells (**Figure 1A** and **1E**), while Matrigel coatings diminished the attachment of both cell lines (**Figure 1C** and **1D**). Therefore, type IV collagen was used to coat the PVA sponge rather than Matrigel, which been originally proposed.

2. Identification of MDA-MB-231 in a HFL-1 / MDA-MB-231 co-culture system.

HFL-1 cells were seeded at 2.5×10^4 in a 24-well plate or 100 mm^2 dish and cultured for 24 hours. Then, 2.5×10^4 MDA-MB-231 cells were added, and growth of the culture was continued for an additional 24 hours. The co-cultured cells were fixed with 4% paraformaldehyde (PFA, Sigma) solution, and then stained by immunocytochemistry according to our previous reports [2, 3]. The primary antibody was a monoclonal mouse anti-human epithelial membrane antigen (EMA) IgG (DAKO Corporation, Carpinteria, CA). The secondary antibody was an alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Sigma Immuno Chemicals, St. Louis, MO). SIGMA FAST^{TM} Fast Red TR/Naphthol AS-MX (Sigma) was used as substrate to detect positive signals, as shown in **Figure 2**.

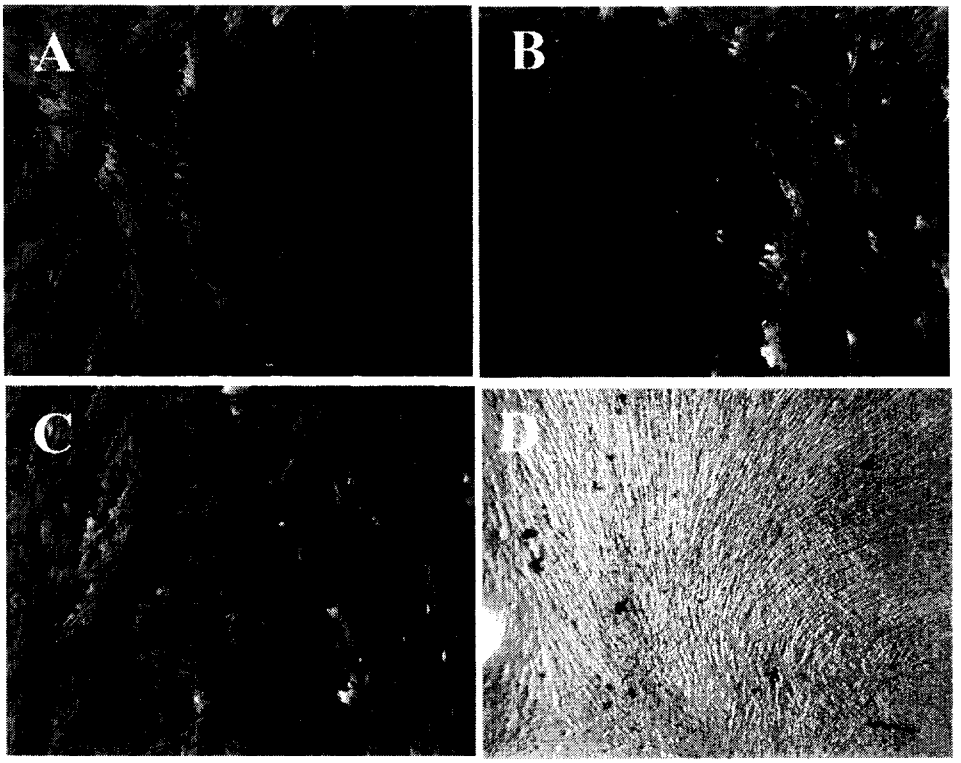

Figure 2. Immunohistochemical staining of epithelial membrane antigen (EMA) in MDA-MB-231 / HFL-1 cell co-culture systems. In this co-culture system, an anti-EMA antibody could be used as a marker to differentiate MDA-MB-231 cells from HFL-1 cells (Figure 2A-C), as the HFL-1 cells demonstrated only background staining signals (Figure 2D). Under normal culture conditions, HFL-1 cells grow evenly, in a 2-dimensional fashion; but when co-cultured with MDA-MB-231 cells, the HFL-1 cells formed 3D-structures, and the MDA-MB-231 cells clustered as pathological tissues.

3. Growth of MDA-MB-231 and HFL-1 cells in PVA sponge.

A great deal of time and energy was devoted to this particular task. Many methods were used to pre-treat the PVA sponge prior to cell seeding, including autoclaving and immersion in 70% ethanol, but both of these methods left the sponge shrunken and deformed. Overnight exposure to UV light resulted in decontamination of only the sponge surface given the thickness of the sponge itself. Finally, the sponge was cut into two parts, soaked in PBS solution with 1% penicillin and streptomycin under UV overnight, then dried it in the hood under UV. Following pre-treatment of the PVA

sponge, the sponge was coated with 100 µl type IV collagen (1:250 dilution) and allowed to dry under UV light. The sponge was then incubated in fresh media for 30 minutes prior to seeding with MDA-MB-231 and HFL-1 cells. Seeding was accomplished by incubating 80 µl MDA-MB-231 or HFL-1 cells ($2.5X10^5$) with a sponge in the autoclaved caps of a 1.5 ml Eppendorf tube. The caps and sponges were then placed into covered 24-well plates and incubated at 37 ° C in 5% CO_2 for 1 hour without adding culture media. The sponge was then moved to a new 24-well plate in the presence of fresh media. The sponge was incubated for 5 days, with replacement of media every two days.

4. Construction of the 3-dimensional (3-D) cell culture system.

To separate the top and bottom layers we utilized a special insert that is slightly raised within the well, supported by three stands, and containing a polycarbonate membrane with 8 µm pores at its base. In this way, the bottom layer of sponge remained in contact with the ECM/BM layer while the upper chamber was seeded with MDA-MB-231 cells. After 48 hours invasion, the bottom layer sponge was separated from the insert and growth was continued for an additional 3 days. The sponge was then washed with PBS and embedded in Optical Cutting Temperature compound, and finally placed into -80 °C for sectioning and mounting onto slides. The thickness of the sections was 50 µm, and standard immunohistochemical staining was performed according to our previous reports [2, 3], and these results are shown in **Figure 3**. The primary antibody was a monoclonal mouse anti-human epithelial membrane antigen (EMA) IgG (DAKO Corporation, Carpinteria, CA).

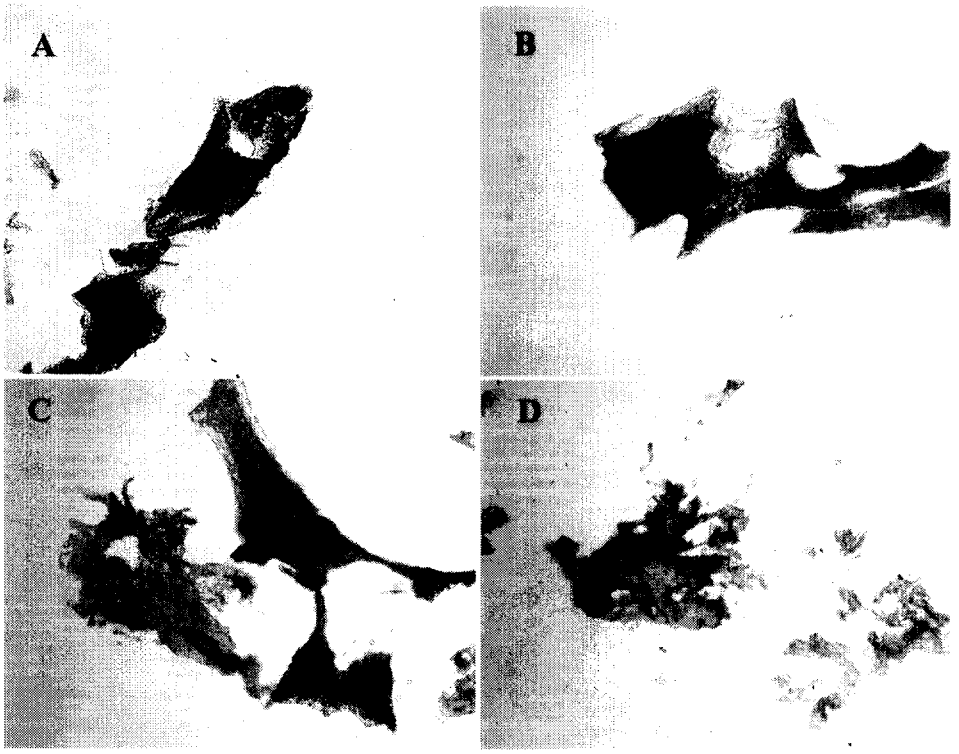

Figure 3. Immunostaining of epithelial membrane antigen (EMA) molecules in the 3D culture system.

In the 3-D culture system, MDA-MB-231 cells were stained red, indicating the presence of EMA, while the underlying HFL-1 cells had only weak staining signals (Figure 3A, 3C, and 3D). Normal mouse IgG was utilized as a control, as shown in Figure 3B. In this 3D-culture system, the MDA-MB-231 cells formed cancer cell clusters similar to cancerous breast tissues (Figure 3C and 3D). Therefore, this model may represent a useful tool to study epithelial-stromal cell interactions and cancer cell invasion in a 3D environment that mimics physiological conditions.

4.1 Difficulties and Solutions:

(1) MDA-MB-231 and HFL-1 cells did not attach well to the PVA sponge, even in the presence of pre-coating with type IV collagen.

Solution: Gelfoam was utilized in addition to PVA, and Gelfoam was found to be promote enhancement superior to that of even the coated PVA sponge.

(2) Cells were not evenly distributed within the PVA sponge, and therefore, the number of invaded cells may not be indicative of invasion through a true 3D environment.

Solution: The sponge was cut into smaller sizes (5×4×3mm), cells were suspended at higher concentrations (1.25×10^8), and care was taken to ensure that the cells were seeded in the center of the sponge. Floatation of the sponge was also deterred by the weight of the media in the upper chamber.

(3). It is very difficult to cut PVA sponge to 10 μm thickness.

A. As the media contained within the PVA sponge could not be completely evacuated prior its being embedded in Optical Cutting Temperature compound, the sponge was broken very easily. Evacuation of all media by squeezing the sponge was not considered, as this would most certainly effect the shape of the cells and their position within the sponge.

B. The pore size of the PVA sponge was too large, and when 10 μm sections were prepared, the result was pieces of sponge as opposed to a full section. It was later determined that 50 μm was the minimum thickness that yielded and intact slide.

C. Even at a thickness of 50 μm, immunohistochemical staining frequently results in dissociation of the sponge from the slide, as shown in **Figure 3D**.

Solution: To solve these difficulties, we are incorporating the Gelfoam sponge into our current investigation in addition to the PVA sponge originally proposed.

5. MMP-26 knockdown in MDA-MB-231 cells by siRNA transfection.

The construction of the siRNA-expression plasmids was based on the siSTRIKE™ U6 Hairpin Cloning Systems (Promega). The vector includes a human U6 promoter, Amp^r / $Neomycin^r$ genes, and facilitated a sticky ends with downstream overhang PstI partial sites. The inserted hairpin sequence, which includes sense nucleotides, a loop-creating region, and anti-sense nucleotides, was designed using the siRNA Target Designer Program (www.promega.com/siRNADesigner/). The sequences produced by this program are compared to all sequences in Genbank using the NCBI BLAST server. Among those sequences, only the specific MMP-26 target sequence was selected, and the control scramble sequence was designed in a similar fashion. The forward and backward sequences of the MMP-26 siRNA target insert and the scramble insert, respectively, are shown below:

5'-ACCGGAAGATGCAAGTGGAATAAAGTTCTCTTATTCCACTTGCATCTTCCTTTTTC -3'

5'-TGCAGAAAAAGGAAGATGCAAGTGGAATAAGAGAACTTTATTCCACTTGCATCTTC -3'

5'-ACCGATAGTGAACGGTAAGAAGAAGTTCTCTCTTCTTACCGTTCACTATCTTTTTC -3'

5'-TGCAGAAAAAGATAGTGAACGGTAAGAAGAGAGAACTTCTTCTTACCGTTCACTAT -3'.

After annealing, the DNA fragments were ligated, producing one new PstI site in addition to the existing PstI site, which were used for selection. Transfection of the MDA-MB-231 cells with MMP-26 siRNA target siSTRIKE™ has resulted in an approximately 90% knock down of MMP-26 expression in this stably transfected cell line when compared to normal MDA-MB-231 cells and the scramble siRNA transfected cell line, as shown in **Figure 4**.

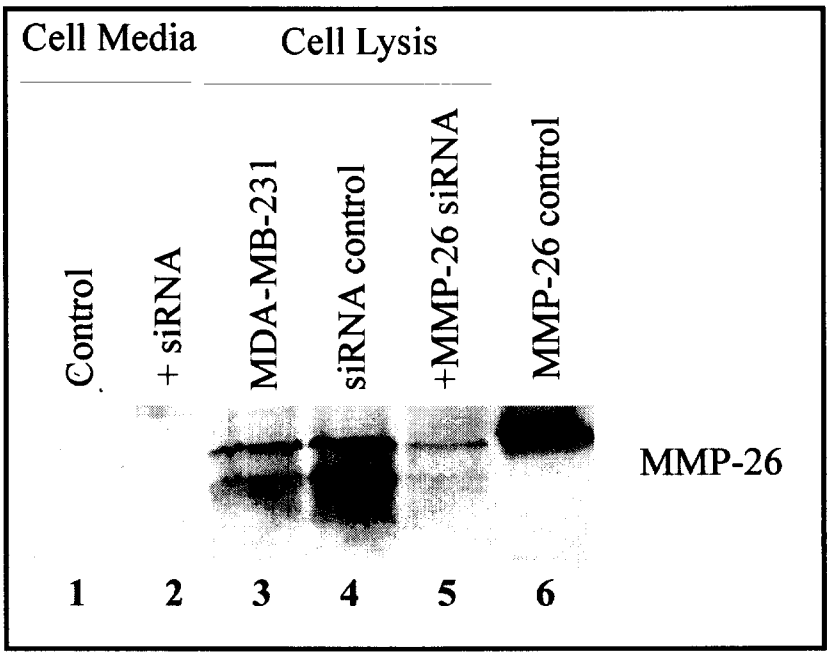

Figure 4. Expression of MMP-26 in normal and siRNA transfected MDA-MB-231 cells. Lanes 1 and 2 are cell culture media from normal MDA-MB-231 cells and MMP-26 siRNA transfected MDA-MB-231 cells, respectively. Lanes 3 to 5 are cell lysates from parental MDA-MB-231 cells, MDA-MB-231 cells transfected with siRNA scramble inserts, and MMP-26 siRNA transfected MDA-MB-231 cells, respectively. Lane 6 contains purified, recombinant MMP-26 expressed in BL21 (DE3)-competent *E. coli* cells. Lanes 3-6 were loaded with equivalent amounts of total protein.

Summary of Project Progression

Task 1. To establish a three-dimensional polyvinyl alcohol (PVA) sponge culture system and to derive optimum culture conditions for the breast cancer cell line MDA-MB-231 and HFL-1 fibroblast cells (Months 1-6):

a. Establish a three-dimensional PVA/Gelfoam sponge culture system (complete).
b. Determine optimal conditions to accommodate the growth of MDA-MB-231 cells in the top layer and HFL-1 human fibroblast cells in the bottom layer (complete).
c. Perform MDA-MB-231 invasion assays in this 3D culture system (ongoing).
d. Evaluate the utility of this three-dimensional PVA/Gelfoam sponge culture system in comparison with the two-dimensional modified Boyden Chamber invasion assay (ongoing).

Task 2. To investigate the role of matrix metalloproteinase-26 (MMP-26) in MDA-MB-231invasion using this 3D culture system (Months 7-12):

a. Perform MB-MDA-231 invasion assays in this 3D culture system in the presence of purified anti-MMP-26 and/or anti-MMP-9 IgGs and pre-immune IgGs (ongoing).
b. Establish a stably transfected line of MDA-MB-231 in which MMP-26 expression has been knocked down (complete).
c. Perform MDA-MB-231 invasion assays in this 3D culture system utilizing stably transfected cell lines in which MMP-26 expression has been significantly diminished (ongoing).

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Key Research Accomplishments

1. **MDA-MB-231 and HFL-1 cells attachment on the different extracellular matrix components was completed.**

2. **The three-dimensional culture system of the MDA-MB-231 and HFL-1 cells was established.**

3. **MDA-MB-231 cells transfected with MMP-26 siRNA was completed.**

Reportable Outcomes

1. **The grant titled "*The Potential Role of Matrix Metalloproteinase-26 in Human Breast Carcinoma Invasion*" was submmited to The Susan G. Komen Breast Cancer Foundation on September 2, 2003.**

2. **Poster entitled "*Inhibition of MDA-MB-435 cell invasion by a synthetic matrix metalloproteinase inhibitor*" was presented at the 44th American Society for Cell Biology Annual Meeting at the Washington Convention Center in Washington DC. Poster # is 2344. December 4-8, 2004.**

3. **Abstract entitled "*The novel three dimensional cell culture system for human breast cancer cancer invasion and cell-cell interaction*" was accepted for poster presentation at Era of Hope 2005 Departmeent of Defense breast Cancer Research Program Meeting at Pennsylvania Convention Center, Pliladophia, Pennsylvania, June 8-11, 2005.**

Conclusions:

1. **MDA-MB-231 and HFL-1 attach differentially to ECM components.**

2. **Epithelial membrane antigen is a suitable marker to distinguish MDA-MB-231 cells from HFL-1 cells in 2D and 3D co-culture systems.**

3. **MMP-26 siRNA transfection was successful in knocking down 90% of the endogenous MMP-26 expression in MDA-MB-231 cells.**